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Effect of oil refinery sludges on the growth and antioxidant system of alfalfa plants

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ABSTRACT

The refining process in the petrochemical industry generates oil refinery sludges, a potentially contaminating waste product, with a high content of hydrocarbons and heavy metals. Faster degradation of hydrocarbons has been reported in vegetated soils than in non-vegetated soils, but the impact of these contaminants on the plants physiology and on their antioxidant system is not well known. In this study, the effect of the addition of petroleum sludge to soil on the physiological parameters, nutrient contents, and oxidative and antioxidant status in alfalfa was investigated. An inhibition of alfalfa growth and an induction of oxidative stress, as indicated by an increase in protein oxidation, were found. Also, the superoxide dismutase isoenzymes, peroxidase, and those enzymes involved in the ascorbate–glutathione cycle showed significant activity increases, parallel to an enhancement of total homoglutathione, allowing plants being tolerant to this situation. This information is necessary to establish successful and sustainable plant-based remediation strategies.

1. Introduction

The regulations to prevent further undue release of hazardous chemicals into the environment have become stricter over the years, and many countries have started to adopt a tighter line on environmental issues. The currently available decontamination methods are expensive, partially due to the cost of excavating and transporting large quantities of contaminated materials for ex situ treatment, such as chemical inactivation or thermal degradation. Because of this and their low-cost, low maintenance, and environmental friendliness, there is an increasing interest in alternative technologies for in situ applications, in particular those based on biological remediation capability of plants and microorganisms [1]. Phytoremediation may be defined as the use of plants to remove, destroy or sequester hazardous substances from soil environment, and it can be applied to both organic and inorganic pollutants, present in soil substrates, liquid substrates, and air [2]. Its main application has been to remove toxic heavy metals form soil [3]. However, there is a growing interest in broadening this technology to remove/degrade organic pollutants in the environment [4].

The petrochemical industry generates a series of liquid effluents during the refining process which must be treated through depuration processes. The results of these processes are oil refinery sludges, potentially a waste product, that have a high content of petroleum-derived hydrocarbons [5]. Organic contaminants can be stabilized within a soil matrix, taken up by plants and transformed or stored in a non-phytotoxic form. Plants can also stimulate the rhizosphere microbial community that is capable of degrading organic contaminants [6]. Thus, faster degradation of petroleum polycyclic aromatic hydrocarbons (PAHs) has been reported in vegetated soils than in non-vegetated soils, and this has been mediated by root-associated microorganisms [7]. However, it is not completely understood how specific plants increase the remediation of contaminated soils. Kirk et al. [8] showed that alfalfa seems to specifically increase the number of microorganisms capable of degrading complex petroleum hydrocarbons, and in several studies, alfalfa and other plants bearing an abundant root system have been shown to phytoremediate or tolerate several aromatic compounds like phenol, benzene and PAHs [9].

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Information on the impact of these contaminants on the plant physiology is quite scarce. Considering that the plant physiological status may directly affect the plant detoxification capacity, it is necessary to assess the effect of the target compound on the plant system. Plants are known to have the ability to respond and adapt to a variety of biotic and abiotic stresses. In soils polluted with organic chemicals, plants may experience a combined stress from nutritional deficiency and chemical toxicity. Reactive oxygen species (ROS) such as the superoxide radical ($O_2^{\bullet -}$), H_2O_2, and hydroxyl radical ($^{\bullet}OH$), are generated as by-products of normal metabolism in different subcellular compartments. Furthermore, the imposition of biotic or abiotic stress may give rise to an excessive concentration of ROS, resulting in oxidative damage at cellular level that can be mitigated and repaired by a complex antioxidant system. On the other hand, plants use ROS as second messenger in many signal transduction cascades and thus ROS accumulation is crucial to plant development and defence. For these reasons the plant antioxidant defence network is important in controlling the life-time of the ROS signals and in preventing uncontrolled oxidation [10–12]. The metalloenzyme superoxide dismutase (SOD), is the major $O_2^{\bullet -}$ scavenger and its enzymatic action leads to H_2O_2 and O_2 formation. H_2O_2 is eliminated by catalase (CAT), by several classes of peroxidases and, by the action of the ascorbate–glutathione cycle, which includes ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) [13,14]. Glutathione (GSH) and ascorbate (ASC) are crucial for plant defence against oxidative stress. ASC is a small, water-soluble antioxidant molecule, used as substrate for APX which then catalyses the H_2O_2 detoxification. ASC also acts directly to eliminate superoxide radicals and $^1O_2$, acting as a secondary antioxidant during reductive recycling of the oxidized form of tocopherol. Oxidized ascorbate is reduced by MDHAR and DHAR, using NADH and GSH respectively as the reducing substrates. Oxidized glutathione (GSSG) is then reduced by GR using NADPH as co-factor, closing the cycle and functioning to prevent the accumulation of H_2O_2. The tripeptide glutathione (GSH; γGlu-Cys-Gly) is the major non-protein thiol in most animals, plants, and prokaryotes, and is involved in many vital functions of plants, in the transport and the storage of reduced sulphur [15], in the detoxification of xenobiotics via glutathione-*S*-transferase [16], in the protection against heavy metals as a precursor in the synthesis of phytochelatins [17], in the scavenging of active oxygen species by the ASC–GSH cycle, and in the regulation of the redox homeostasis of the cell. Another thiol tripeptide, homoglutathione (hGSH; γGlu-Cys-βAla), is present in nodules and other organs of some legume species in addition to or in place of GSH (γGlu-Cys-Gly) [18] and may share with it some antioxidative and regulatory properties [19].

The antioxidant system and its significance for the acclimation of plants to air pollution and climatic stresses has been reviewed frequently [20], although little is known about the effects of petroleum sludge stress on the growth and on the antioxidative system in plant, since these growth conditions may produce some oxidative effects in these plants. The effect of petroleum sludge mixed with soil, on the growth and mineral content of alfalfa plants was investigated in this study. In addition, the levels of lipid peroxidation and protein oxidation as well as the antioxidant contents and antioxidant enzyme activity of alfalfa leaves were also analyzed. Alfalfa plants were selected based on previous research showing their phytoremediation potential and their ability to germinate and grow in a petroleum-contaminated soil [21]. The information resulting from this study may help to understand better the mechanisms involved in the observed acclimatization of these leguminous plants to growth in soils with high content of this type of organic pollutants and so establish successful and sustainable plant-based remediation strategies.

2. Material and methods

2.1. Material

The organic material consisted of a sludge generated in an oil refinery (REPSOL-YPF) in SE Spain, which has a very high hydrocarbon content, about $336 \pm 10\,g\,kg^{-1}$ and presents similar characteristics to that described by Marín et al. [22]. This sludge was mixed with soil to give a proportion of 4–5% hydrocarbons, the best one for degradation processes [5]. Duplicated plots were designed for the different treatments in Cartagena, Murcia (SE Spain), near the refinery. Plots of 3 m × 3 m were prepared for soil (control) and soil mixed with sludge (+sludge). The plant material used was alfalfa (*Medicago sativa* L.) seeds, which were purchased from the local market (Ramiro Arnedo S.A.) and were spread onto the plots (90 g of seeds per plot).

2.2. Sample processing

Soil samples from different places of each plot were bulked, mixed, air dried and sieved through 10-mm screen. The soil sample was placed in polyethylene bags, closed and stored at 4 °C prior to analysis. Plants were harvested after nine weeks of growing. Plant growth was determined by measuring fresh weight of the root and the aerial part, and length of shoots and roots. 12 plants per plot were randomly collected and rapidly frozen with liquid nitrogen and stored at −80 °C until analysis.

2.3. Analysis of mineral content

Dried plant material and soils with and without sludge were used to measure the mineral content as described in Marín et al. [22]. P was determined by colorimetry and K, Ca, Mg and heavy metals by atomic absorption. Total organic C and total N were determined by elemental analyzer. Total petroleum hydrocarbon content was measured by the infrared EPA method no. 8440 [23].

2.4. Lipid peroxidation and protein oxidation

The extent of lipid peroxidation in leaves was estimated by determining the concentration of thiobarbituric acid reactive substances (TBARS) [24]. Protein oxidation (carbonyl protein content) was measured by reaction with 2,4-dinitrophenylhydrazine, as described by Levine et al. [25].

2.5. Leaf enzyme extraction

The leaf tissue was homogenized according to Camejo et al. [26]. For APX activity, sodium ascorbate (20 mM) was included and EDTA was omitted in the extraction buffer, which consisted of 50 mM Hepes–NaOH (pH 7.0). Protein was measured by the protein dye-binding method of Bradford [27] using bovine serum albumin as standard.

2.6. Enzyme activity assays

Total peroxidase (POD) (EC 1.11.1.7) was analyzed as described by Ranieri et al. [28]. Ascorbate peroxidase (EC 1.11.1.11), monodehydroascorbate reductase (EC 1.6.5.4), dehydroascorbate reductase (EC 1.8.5.1), glutathione reductase (EC 1.6.4.2), and superoxide dismutase (EC 1.15.1.1) activities were assayed according to previously published protocols by Jiménez et al. [29]. Enzyme activities were corrected for non-enzymatic rates and for interfering oxidations. To separate and identify SOD isoenzymes, non-denaturing PAGE and isoelectrofocusing were performed like previously described

Camejo et al. [26]. The isoenzymes activities were quantified on an image analyzer (Gen Tools, Syngene).

2.7. Determination of glutathione and related compounds

Leaf tissue extraction and analysis by liquid chromatography–electrospray/mass spectrometry were performed as described by Rellán-Álvarez et al. [30] to determine reduced and oxidized GSH and hGSH. Briefly, plant tissue (100–500 mg) was ground with mortar and pestle in liquid N_2. Labelled GSH ([glycine 1,2-^{13}C,^{15}N]GSH) was added at the moment of sample grinding. The dry powder was homogenized with cold extraction solution (5% (w/v) m-phosphoric acid and 1 mM EDTA in 0.1% formic acid), supplemented with 1% (m/v) polyvinyl-polypyrrolidone (PVPP) just before use. Homogenates were centrifuged at 15 000 × *g* for 20 min at 4 °C and supernatants were filtered through 0.22-μm polyvinylidene fluoride filters, frozen in liquid N_2 and stored at −80 °C until analysis. Analyses were carried out with a BioTOF II (Bruker Daltonics, Billerica, MA, USA) coaxial multipass time-of-flight mass spectrometer (MS(TOF)) equipped with an Apollo electrospray ionization source (ESI) and coupled to a Waters Alliance 2795 HPLC system (Waters, Milford, MA, USA).

2.8. Determination of ascorbate and dehydroascorbate

Ascorbate was extracted from leaf tissue using 10% m-phosphoric acid and incubating with ice in the dark for 30 min. The mixture was diluted with distilled water to give a final concentration of 5% m-phosphoric acid and centrifuged at 15 000 × *g* for 10 min. ASC and dehydroascorbate (DHA) in the supernatant were determined immediately by HPLC. DHA was quantified by incubating the samples for 24 h at room temperatures with 1 mM dithiothreitol (DTT). The DHA concentration was measured as ASC after rechromatography [31].

2.9. Statistical analysis

The experiment was conducted in a completely randomized design. Soil nutrients and nutrient content in plants are the mean of at least three replicates and plant growth results are the means of 12 replicates per treatment (six per plot). The rest of the parameters are the mean of six independent replicates of each plant treatment (three per plot). Data were analyzed and compared by the Student's *t*-test.

3. Results and discussion

3.1. Nutrient content of soil

The sludge used in this work comes from the refinery of REPSOL-YPF in Murcia (Spain) and is characterised by a high hydrocarbons content (336 ± 10 g kg^{-1}), and is therefore similar to that used by Marín et al. [5]. Phenol is one of the main organic compounds in the sludge, which also contains high amounts of Zn, Pb, Ni, Cu, and Cr although they are under the limits established by the EU legislation for the use of sewage sludge in agriculture [32]. All of them have been described as being able to induce oxidative stress and different harmful effects on plant metabolism depending on their concentrations [33]. The soil, in which the experiment was carried out, is poor in organic matter and nutrients and has a metal content that reflects its proximity to a mining area [5]. These authors described the bioremediation of the oil refinery sludge by landfarming, studying its influence on the soil microbial activity. In this study, it was reported that 80% of biodegradation of hydrocarbons presented in the oil sludge occurred in 11 months, and half of this reduction took place in the first three months. For this reason, we have chosen this moment (three months) to sow the alfalfa seeds. Plants were taken nine weeks later, when the growth period was sufficient to reflect the degree of their acclimatization.

Table 1
Nutrient content in soil.

		Control	+Sludge
C	g 100 g^{-1}	1.2	2.5
N		0.16	0.27
K		0.71	0.59
Ca		13.5	13.3
Mg		1.1	1.0
P	mg kg^{-1}	712.3	875.9
Fe		12.1	15.0
Mn		251.8	253.1
Cu		26.3	40.6
Zn		44.9	150.6
Cd		<2.5	<2.5
Pb		5.3	15.5
Ni		16.2	35.3
Cr		6.4	14.0

Results from the macronutrients, micronutrients and heavy-metals analysis, verified that the soil in the plots with sludge contained hydrocarbons in a proportion of around 4% and elevated contents of P, Zn, Cu, Pb, Ni, and Cr when compared with the control soil (Table 1), although the heavy-metals content in these soil was within the normal range found in different soils [34]. Zinc was the highest-polluting element in the sludge, with available zinc in soil with sludge at 150.6 mg kg^{-1}, which was inside the toxic range for plants (70–400 mg kg^{-1}) [35]. Copper was another polluting element in the oil refinery sludge, with a concentration of 40.6 mg kg^{-1} when it was mixed with soil, which was above the median of normal soil but was out of the toxic range for plants (60–125 mg kg^{-1}) [35]. Lead is a toxic heavy metal that causes numerous health problems and its excess in soils is a major concern in industrialized countries. Pb concentration was found to increase around 3-fold in soils with added sludge from the refinery, although the final concentration (15.5 mg kg^{-1}) was under the accepted threshold (100 mg kg^{-1}) for toxic effects in plants [35]. Nickel and chrome in the sludge were also high but in the mixture they were below established toxicity levels in soil for plants (100 mg kg^{-1} for Ni and 75 mg kg^{-1} for Cr) [35]. Cadmium was not a polluting element in the sludge. The degradation of complex mixtures of contaminants like those present in the petroleum hydrocarbons is quite difficult, although there are examples in the literature reporting that the disappearance of several PAHs is faster in soils planted with a mixture of prairie grasses or ryegrass than in unvegetated soils [36]. In our work, the presence of alfalfa in the sludge contaminated soils did not modify the total carbon content in the soil (data not shown) when compared with a contaminated soil without plants, indicating that alfalfa at this time probably did not affect the hydrocarbon degradation. Alfalfa plants have been reported to decrease the phenanthrene concentration in soils [37] but other reports describe that of twenty genotypes evaluated, only two resulted in a decrease in total petroleum hydrocarbon concentration in soil after 12 months [38], so the heritability of phytoremediation for alfalfa needs further investigation.

3.2. Physiological parameters and nutrients content in alfalfa plants

Biological remediation is often slower than physical or chemical degradation, and this is particularly evident in the case of plants, where the growth is dependent on environmental factors. Many plant species have been chosen to clean up contaminated soils but it is very important to determine the toxicity of the contaminants towards the plant before starting a phytoremediation project. In order to provide the vegetable cover and to create an adequate rhi-

Table 2
Influence of petroleum sludge on plant development. Values are mean ± SE, $n = 12$. Differences are significant at level $p < 0.05$ (*), $p < 0.01$ (**).

	Control	+Sludge
Aerial Fwt (g)	8.9 ± 1.8	6.5 ± 1.2
Root Fwt (g)	1.9 ± 0.4	1.5 ± 0.2
Shoot length (cm)	52.7 ± 2.1	33.6 ± 0.9*
Root length (cm)	26.4 ± 3.5	16.4 ± 0.9**

zosphere for the microbial community, alfalfa plant was chosen for providing a very cheap and abundant biomass and for its good characteristics of culture: few soil and climatic requirements and good resistance to adverse climatic conditions, growing easily in semiarid regions as the SE Spain.

Alfalfa germination has been reported to be retarded in presence of 100 mg kg^{-1} of diesel [39], and in our experiment, the percentage of germination decreased about 15% when compared with control soil. The presence of sludge also affected growth of alfalfa plants (Table 2), with the shoot and root length being significantly higher, about 1.6-fold, in control soil. The fresh weight of aerial part and roots were also diminished by the presence of sludge but in a non-significant way. Similarly, Kirk et al. [8] showed that alfalfa germinated in petroleum-contaminated soil but growth was stressed and stunted as has been shown in *Astragalus adsurgens* [40]. The results on the growth response obtained in our experiment, indicated that alfalfa plants are a good choice to develop remediation assays, since although growth was affected, the plants appeared healthy and continued their normal development.

The analysis of the nutrient content in plants (Table 3) showed that the concentration of macronutrients in roots, shoots and leaves of plants grown in both, control and contaminated soils was similar, except for K and Na in root and P in shoot and root, which were present in higher concentrations; 1.8-fold, 2.3-fold, and 1.7-fold, respectively, in plants grown in contaminated soil. Although essential for plant metabolism, micronutrients can produce toxic effects if they reach very high concentrations in the plant tissues. The addition of sludge to soil increased the concentration of Mn 1.8-fold in shoot and 1.2-fold in leaves, while Fe increased only 1.2-fold in leaves and decreased in roots and shoots. Zn, which was the highest-polluting element in the sludge, increased its concentration 1.9-fold in shoot and 1.6-fold in leaves and root, although plant Zn concentration was within the normal range in plants [34]. These results indicated that Zn was the most mobile element in terms of translocation within plant tissues, as previously described by Benzarti et al. [41] for alfalfa plants grown in nutrient solution with added Cd, Zn, and Cu. These authors also showed that plant growth was significantly affected by metal concentration elevation in the solutions, with Zn being the less phytotoxic metal. In spite of their increased concentration in soil with sludge, Cu, Ni, and Cr did not change their accumulation tendency in presence of sludge, and their levels were below the phytotoxic concentration in plants. The alfalfa plants presented low Pb concentration in leaves, shoots, and roots (<5 mg kg^{-1}) which were far below the suggested toxicity level for livestock (30 mg kg^{-1}). Pb is considered to have low availability for plant uptake and alfalfa is not considered a heavy-metal hyperaccumulating species but some interesting experiments using EDTA and some phytohormones in combination with Pb have shown that Pb increased its concentration in the alfalfa leaves [42], demonstrating that a non-hyperaccumulating plant like alfalfa could accumulate Pb in adequate conditions.

Table 4
Petroleum sludge effects on lipid peroxidation and protein oxidation in alfalfa leaves. Values are mean ± SE, $n \geq 6$. Differences are significant at level $p < 0.05$ (*).

	Control	+Sludge
Lipid peroxidation (μmol ml^{-1})	193.0 ± 4.7	149.5 ± 18.7
Protein oxidation (nmol mg^{-1} prot)	11.3 ± 0.9	22.3 ± 2.0*

3.3. Oxidative stress and antioxidants in alfalfa leaves

Evidence accumulated from different plant systems show that they are able to respond to increased ROS with enhanced synthesis or activity of some antioxidant enzymes that could partly counteract their accumulation. However, this increased antioxidant capacity is not always produced, and the intensity of response is dependent on the plant species and tissue analyzed, the stressor and the intensity of the stress. It has been frequently reported that exposure of plants to excess concentration of some heavy metals, usually results in oxidative injury and in modifications of some components of the antioxidant systems [43,44]. However, little is known about the simultaneous effect of sludge on plant development and on the response of its antioxidants.

Protein carbonylation is an irreversible oxidative process leading to a loss of function and often proteolysis of the modified proteins. Analysis of protein oxidation is an approach to reflect oxidative stress and damage caused to cells during different environmental stresses. Significant increase (about 2-fold) in the level of oxidized proteins was found in leaves of alfalfa plants grown in the presence of petroleum sludge. By contrast, the level of another oxidative process, lipid peroxidation, did not change significantly (Table 4), indicating that proteins were more susceptible to the oxidative modifications than lipids in alfalfa under these growth conditions.

Table 3
Influence of petroleum sludge on plant nutrient concentration and heavy-metals content.

		Leaves		Roots		Shoots	
		Control	+Sludge	Control	+Sludge	Control	+Sludge
C		51.0	52.0	55.4	55.3	52.9	53.2
N		5.6	5.9	2.6	2.6	2.0	2.1
K		0.77	0.86	0.57	1.03	1.08	0.92
Ca	g 100 g^{-1}	1.01	0.83	0.15	0.22	0.34	0.23
Mg		0.13	0.13	0.09	0.09	0.10	0.08
P		0.12	0.14	0.06	0.10	0.06	0.11
Na		0.14	0.15	0.07	0.16	0.08	0.12
Ni		6.1	5.7	4.9	5.5	6.5	6.5
Cu		8.0	7.5	8.1	7.5	7.1	6.5
Zn		19.5	31.2	14.5	25.5	11.1	21.0
Fe	mg kg^{-1}	156.4	182.7	248.4	213.9	120.9	80.2
Mn		26.0	31.5	8.5	7.0	3.0	5.5
Cd		<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
Pb		<1.0	<1.0	2.1	2.3	2.7	<1.0
Cr		<5.0	<5.0	<5.0	<5.0	<5.0	<5.0

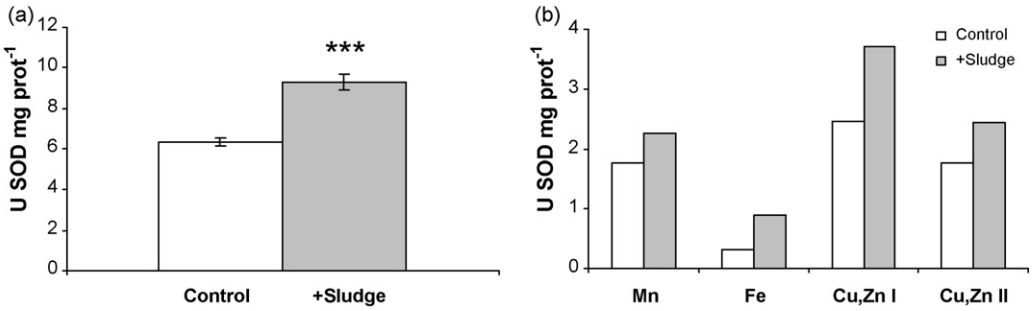

Fig. 1. Changes in superoxide dismutase (SOD) activity (U mg^{-1} protein) of alfalfa leaves in response to petroleum sludge. (a) Total SOD, (b) SOD isoenzymes. Values are mean ± SE, $n \geq 6$. Differences are significant at level $p < 0.001$ (***).

This pattern is similar to that previously described by Escuredo et al. [45] and Gogorcena et al. [46] in legume nodules induced to senesce by exposure to nitrate or darkness.

SOD specific activity was measured in leaves of alfalfa plants, and it was found to increase in the presence of sludge (Fig. 1a). The analysis of the SOD isoenzymes, allowed to detect five different isoenzymes in leaves extracts: a slow moving Mn-SOD, a Fe-SOD, and three Cu, Zn-SODs, as was described by McKersie et al. [47] in alfalfa. Interestingly, in plants grown in the presence of sludge, an induction in the activity of all the SOD isoenzymes was produced (Fig. 1b), that could be favoured by the elevated concentrations of Zn, Fe, and Mn found in leaves. Similar to that, the induction of a Mn-SOD in leaves of pea plants grown under high nutrient levels of Zn and Mn has been reported [43]. SOD induction has been widely described in different plant species in response to heavy-metal stress [12,48], however the effects on SOD activity in plants depend on the species, tissue and experimental conditions. Increased SOD activity has a number of other physiological effects. Apart from preventing superoxide accumulation, it is expected to enhance the H_2O_2 content thus a balance between its production and elimination must exit to avoid increased ROS oxidative damage. In this sense, the observed induction of SOD activity ran parallel to a significant increase in the specific activity involved in the H_2O_2 elimination through POD, but not in the APX activity, which did not change significantly (Table 5). The POD level and isoenzyme pattern can be altered by environmental stresses, so this activity has been used as non-specific biomarker of environmental pollution [49]. Increased peroxidase levels, together with its role in peroxidation protection may also be involved in the detoxification of phenol through its immobilization or the formation of insoluble polymers and in this sense, Flocco et al. [33] showed that the activity of soluble peroxidases of alfalfa roots increased in the presence of 100 mg l^{-1} of phenol in hydroponic culture, while this parameter and its removal capacity were negatively affected in the case of plants exposed to 500 mg l^{-1}. The increase on POD activity found in alfalfa plants grown in presence of sludge would respond to the level of phenol presented in the sludges.

As found for the SOD isoenzymes and POD, the activity of all the enzymes involved in the ASC–GSH cycle showed significant increases in leaves of plants grown in presence of sludge (Table 5), which indicates that under this condition the cellular capacity to maintain cellular ASC and GLU pools in their reduced forms through this pathway was not limited. This coordinated increase in the activities was also found by Prasad et al. [50] in shoots of seedlings of *Brassica juncea* raised in the presence of toxic levels of zinc, similar to that occurring in our experiment conditions. A similar coordinated induction in the activity of the different antioxidative enzymes has been reported to be important in the tolerance to different abiotic stress conditions [26,51,10].

Ascorbate and glutathione are key non-enzymatic antioxidants in plants. They are involved in the ASC–GSH cycle, an important part of the H_2O_2 detoxification pathway [52,14] and they also function as independent redox signalling molecules. Both compounds make up the major redox buffer in the plant cell, and there are considerable evidence for the central importance of ASC in plant biology, with roles in hormone synthesis, gene expression, cell division, growth, and apoptosis [53]. When the ascorbate content was analyzed, we found that the total concentration was similar in leaves from plants grown in both types of soils (Table 6). Interestingly, the ASC/DHA ratio was increased in plants grown in soils contaminated with sludge (Table 6), due to a decreased in their DHA, which agrees well with the enhancement of MDHAR, DHAR, and GR activities found in these plants. The maintenance of total ascorbate level (Table 6) indicates that its content is determined by the relative rate of synthesis and degradation, which under these growth conditions seems to be compensated. These results on ASC/DHA ratio and ASC level may favour that under these conditions there was not a higher oxidative stress.

Like ASC, GSH is a multifunctional compound with important functions outside the antioxidant system [13]. In this study, we found that total glutathione level was similar in both plants and that the GSH/GSSG ratio was not affected (Table 6). On the other hand, together with GSH, some legume species contain an homolog thiol tripeptide, the hGSH. All these compounds share an equivalent chemical structure, which points to similar functions in plants [54]. In this work we have observed that hGSH in alfalfa leaves was more abundant (about 50-fold higher) than GSH, as previously described by Matamoros et al. [18]. When the homoglutathione

Table 5
Changes in ascorbate–glutathione cycle enzymes activity (nmol mg^{-1} protein) in alfalfa leaves, in response to petroleum sludge. Values are mean ± SE, $n \geq 6$. Differences are significant at level $p < 0.01$ (**).

	Control	+Sludge
POD	381 ± 7.0	445 ± 13.7**
APX	154.8 ± 44.7	105.4 ± 20.0
MDHAR	115.8 ± 3.8	143.9 ± 7.1**
DHAR	28.5 ± 2.9	41.0 ± 1.8**
GR	26.3 ± 0.8	42.2 ± 3.4**

Table 6
Changes in the non-enzymatic antioxidants ascorbate (ASC), glutathione (GSH), and derivatives of alfalfa leaves in response to petroleum sludge. Values are mean ± SE, $n \geq 6$.

		Control	+Sludge
ASC	μg g^{-1} Fwt	176.1 ± 17.6	185.8 ± 9.9
DHA		26.0 ± 2.9	18.7 ± 2.0
GSH	nmol g^{-1} Fwt	8.4 ± 1.7	8.0 ± 2.3
GSSG		2.0 ± 0.2	1.7 ± 0.2
hGSH		449.6 ± 14.6	501.3 ± 26.7
hGSSG		26.2 ± 3.9	40.3 ± 8.1

content was analyzed, we found that the total concentration was higher in plant leaves grown in presence of sludge (Table 6) showing that the synthesis of homoglutathione was induced in the presence of the contaminant. Also, there was a non-significant change in the hGSH/hGSSG ratio, thus the plants grown in contaminated soils showed a lower ratio than plants grown in control soils (Table 6), which indicates an hGSSG accumulation probably because the induced GR activity is insufficient to maintain the homoglutathione pool in its reduced form under these conditions. Thus, it is clear that in spite of the activation of hGSH synthesis, the accumulation of hGSSG reflects its higher sensitivity to be oxidized under these conditions. The preferential oxidation of GSH, in comparison to ASC is in line with the GSH redox couple having a lower redox potential than the ASC [52]. The highly reduced glutathione pool maintained by GR is necessary for active protein function and avoids unspecific formation of mixed disulphide bonds that cause protein inactivation or aggregation. Although the hGSH level increased in alfalfa plants, a rapid ROS mediated oxidation of hGSH could take place in leaves during sludge growth conditions, so the oxidation observed in leaves proteins under sludge might be favoured by the low hGSH/hGSSG found in these plants. However, an important lipid peroxidation increase was not found, by which we interpreted this response to mean that the ASC–GSH cycle plays an important role in the elimination of H_2O_2 in plants under these growth conditions, which may favour plants grown on sludge not presenting a higher important oxidative stress. Apart from the well documented roles of antioxidative enzymes and antioxidants in removing H_2O_2 and other ROS, the importance of glutathione system in relation to others components of the photoprotective and antioxidative defence system should be taken into account. There is a strong interaction between oxidants and antioxidants at the level of gene expression and translation. This implies that there is considerable overlap in the signal transduction cascades that induce GSH synthesis and those involved in defence functions that use GSH, such as glutathione-*S*-transferases (GST) whose primary biochemical function is conjugation, either of xenobiotics or of intermediates and secondary metabolites [55]. On the other hand, the increased demand for GSH in response to metal-induced oxidative stress can be accounted for by activation of pathways involved in sulphur assimilation and cysteine biosynthesis [56]. Plants also contain heavy-metal-binding peptides termed phytochelatins (PCs) (or homo-phytochelatins (h-PC) in leguminous) whose chemical structure suggests that they are not formed as direct results of expression of a metal tolerance gene, but rather as a products of a biosynthetic pathway, with GSH (or hGSH) being the most likely precursor [17,57]. Pb^{2+} and Zn^{2+} whose concentrations increased in sludge, have been reported to be two of the most active metals ions in provoking PC synthesis in plants when exposing plant cells at non-toxic concentrations [58].

4. Conclusions

The presence of sludge in soil induces in alfalfa plants an oxidative stress, as indicated by an increase in protein oxidation, with the alfalfa plants being tolerant to this situation. The oxidative stress could be provoked by the direct toxicity of the petroleum sludge in the soil and/or a result of the contaminant properties that alter the physical and chemical properties of the soil, potentially affecting oxygen transfer, available water uptake, and nutrient mobility. Our data support the idea that contaminants from the petroleum sludge can be used to establish a vegetated cover, and the increased tolerance in plants to the presence of this hazardous material can be achieved through increased tolerance of oxidative stress, enhancing antioxidant enzyme activity and avoiding significant sludge-induced oxidation of their ascorbate and (homo)glutathione pools.

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References

[1] I. Aranda-Olmedo, P. Marin, J.L. Ramos, S. Marques, Role of the ptsN gene product in catabolite repression of the *Pseudomonas putida* TOL toluene degradation pathway in chemostat cultures, Appl. Environ. Microbiol. 72 (2006) 7418–7421.
[2] Z. Yanqun, L. Yuan, C. Jianjun, C. Haiyan, Q. Li, C. Schvartz, Hyperaccumulation of Pb, Zn and Cd in herbaceous grown on lead-zinc mining area in Yunnan, China, Environ. Int. 31 (2005) 755–762.
[3] C. Garbisu, I. Alkorta, Phytoextraction: a cost-effective plant-based technology for the removal of metals from the environment, Bioresour. Technol. 77 (2001) 229–236.
[4] P. Van Dillewijn, J.L. Couselo, E. Corredoira, A. Delgado, R.M. Wittich, A. Ballester, J.L. Ramos, Bioremediation of 2,4,6-trinitrotoluene by bacterial nitroreductase expressing transgenic aspen, Environ. Sci. Technol. 42 (2008) 7405–7410.
[5] J.A. Marín, T. Hernández, C. García, Bioremediation of oil refinery sludge by landfarming in semiarid conditions: influence on soil microbial activity, Environ. Res. 98 (2005) 185–195.
[6] S.D. Siciliano, J.J. Germida, K. Banks, C.W. Greer, Changes in microbial community composition and function during a polyaromatic hydrocarbon phytoremediation field trial, Appl. Environ. Microbiol. 69 (2003) 483–489.
[7] Q. Chaudhry, M. Blom Zandstra, S. Gupta, E.J. Joner, Utilising the synergy between plants and rhizosphere microorganisms to enhance breakdown of organic pollutants in the environment, Environ. Sci. Pollut. Res. 12 (2005) 34–48.
[8] J.L. Kirk, J.N. Klironomos, H. Lee, J.T. Trevors, The effects of perennial ryegrass and alfalfa on microbial abundance and diversity in petroleum contaminated soil, Environ. Pollut. 133 (2005) 455–465.
[9] S.P. Pradhan, J.R. Conrad, J.R. Paterrek, V.J. Srivastava, Potential of phytoremediation for treatment of PAHs in soil at MGP sites, J. Soil Contam. 7 (1998) 467–480.
[10] J.M. Gómez, A. Jiménez, E. Olmos, F. Sevilla, Location and effects of long-term NaCl stress on superoxide dismutase and ascorbate peroxidase isoenzymes of pea (*Pisum sativum* cv. Puget) chloroplasts, J. Exp. Bot. 55 (2004) 119–130.
[11] A.J. Meyer, The integration of glutathione homeostasis and redox signalling, J. Plant Physiol. 165 (2008) 1390–1403.
[12] M.C. Romero-Puertas, F.J. Corpas, M. Rodríguez-Serrano, M. Gómez, L.A. Del Río, L.M. Sandalio, Differential expression and regulation of antioxidative enzymes by cadmium in pea plants, J. Plant Physiol. 164 (2007) 1346–1357.
[13] C.H. Foyer, A. Trebst, G. Noctor, Protective and signalling functions of ascorbate, glutathione and tocopherol in chloroplasts, in: B. Demmig-Adams, W.W. Adams (Eds.), Advances in Photosynthesis and Respiration, vol. 19, Photoprotection, Photoinhibition, Gene Regulation, and Environment, Kluwer Academic Publishers, Dordrecht, The Netherlands, 2005, pp. 241–268.
[14] A. Jiménez, J.A. Hernández, L.A. Del Río, F. Sevilla, Evidence for the presence of the ascorbate–glutathione cycle in mitochondria and peroxisomes of pea (*Pisum sativum* L.) leaves, Plant Physiol. 114 (1997) 275–284.
[15] S. Kopriva, H. Rennenberg, Control of sulphate assimilation and glutathione synthesis: interaction with N and C metabolism, J. Exp. Bot. 55 (2004) 1831–1842.
[16] D.P. Dixon, A. Lapthorn, R. Edwards, Plant glutathione transferases, Genome Biol. 3 (2002) 3004.1–3004.10.
[17] C.S. Cobbett, Phytochelatins and their roles in heavy metal detoxification, Plant Physiol. 123 (2000) 825–832.
[18] M.A. Matamoros, L.M. Baird, P.R. Escuredo, D.A. Dalton, F.R. Minchin, I. Iturbe-Ormaetxe, M.C. Rubio, J.F. Morán, A.J. Gordon, M. Becana, Stress-induced legume root nodule senescence: physiological, biochemical, and structural alterations, Plant Physiol. 121 (1999) 97–111.
[19] P. Frendo, J. Harrison, C. Norman, M.J.H. Jiménez, G. Van de Sype, A. Gilabert, A. Puppo, Glutathione and homoglutathione play a critical role in the nodulation process of *Medicago truncatula*, Mol. Plant Microbe Interact. 18 (2005) 254–259.
[20] H. Rennenberg, F. Loreto, A. Polle, F. Brilli, S. Fares, R.S. Beniwal, A. Gessler, Physiological responses of forest trees to heat and drought, Plant Biol. 8 (2006) 556–571.
[21] K.A. Reilley, M.K. Banks, A.P. Schwab, Organic chemicals in the environment: dissipation of polycyclic aromatic hydrocarbons in the rhizosphere, J. Environ. Qual. 25 (1996) 212–219.
[22] J.A. Marín, J.L. Moreno, T. Hernández, C. García, Bioremediation by composting of heavy oil refinery sludge in semiarid conditions, Biodegradation 17 (2006) 251–261.
[23] E.P.A., Total recoverable petroleum hydrocarbons by infrared spectrophotometry, E.P.A. method no. 8440 (1996).

[24] I. Cakmak, W.J. Horst, Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase and peroxidase activities in root tips of soybean (*Glycine max*), Physiol. Plant 83 (1991) 463–468.
[25] R.L. Levine, D. Garland, C. Oliver, A. Amici, I. Climent, A. Lenz, B. Ahn, S. Shaltiel, E.R. Stadtman, Determination of carbonil content in oxidatively modified proteins, Methods Enzymol. 186 (1990) 464–478.
[26] D. Camejo, M.C. Martí, E. Nicolás, J.J. Alarcón, A. Jiménez, F. Sevilla, Response of superoxide dismutase isoenzymes in tomato plants (*Lycopersicon esculentum*) during thermo-acclimation of the photosynthetic apparatus, Physiol. Plant. 131 (2007) 367–377.
[27] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Ann. Biochem. 72 (1976) 248–254.
[28] A. Ranieri, F. Petacco, A. Castagna, G.F. Soldatini, Redox state and peroxidase system in sunflower plants exposed to ozone, Plant Sci. 159 (2000) 159–168.
[29] A. Jiménez, J.A. Hernández, G. Pastori, L.A. Del Río, F. Sevilla, Role of the ascorbate–glutathione cycle of mitochondria and peroxisomes in the senescence of pea leaves, Plant Physiol. 118 (1998) 1327–1335.
[30] R. Rellán-Álvarez, L.E. Hernández, J. Abadia, A. Álvarez-Fernández, Direct and simultaneous determination of reduced and oxidized glutathione and homoglutathione by liquid chromatographyelectrospray/mass spectrometry in plant tissue extracts, Anal. Biochem. 356 (2006) 254–264.
[31] H. Vanacker, L.M. Sandalio, A. Jiménez, J.M. Palma, F.J. Corpas, V. Meseguer, M. Gómez, F. Sevilla, M. Leterrier, C.H. Foyer, L.A. Del Río, Roles for redox regulation in leaf senescence of pea plants grown on different sources of nitrogen nutrition, J. Exp. Bot. 5 (2006) 1735–1745.
[32] C.E.C., Council directive on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture, Off. J. Eur. Commun. L181 (1986) 6–12.
[33] C.G. Flocco, A. Lo Balbo, M.P. Carranza, A.M. Giulietti, Removal of phenol by alfalfa plants (*Medicago sativa* L.) grown in hydroponics and its effect on some physiological parameters, Acta Biotechnol. 22 (2002) 43–54.
[34] H.J.M. Bowen, Environmental Chemistry of the Elements, Academic Press, London, 1979.
[35] A. Kabata-Pendias, H. Pendias, Trace Elements in Soils and Plants, CRC Press, Boca Ratón, Florida, 1992.
[36] W. Aprill, R.C. Sims, Evaluation of the use of prairie grasses for stimulating polycyclic aromatic hydrocarbon treatment in soil, Chemosphere 20 (1990) 253–265.
[37] N. Wu, S. Zhang, H. Huang, P. Christie, Enhanced dissipation of phenanthrene in spiked soil by arbuscular mycorrhizal alfalfa combined with a non-ionic surfactant amendment, Sci. Total Environ. 394 (2008) 230–236.
[38] C.C. Wiltse, W.L. Rooney, Z. Chen, A.P. Schwab, M.K. Banks, Greenhouse evaluation of agronomic and crude oil phytoremediation potential among alfalfa genotypes, J. Environ. Qual. 27 (1998) 169–173.
[39] Z. Al-Ghazawi, I. Saadoun, A. Al-Shak'Ah, Selection of bacteria and plant seeds for potential use in the remediation of diesel contaminated soils, J. Basic Microbiol. 45 (2005) 251–256.
[40] L. Xin, L. Xiaojun, L. Peijun, L. Fei, Z. Lei, Z. Qixing, Evaluation of plant–microorganism synergy for the remediation of diesel fuel contaminated soil, Bull. Environ. Contam. Toxicol. 81 (2008) 19–24.
[41] S. Benzarti, S. Mohri, Y. Ono, Plant response to heavy metal toxicity: comparative study between the hyperaccumulator *Thlaspi caerulescens* (Ecotype Ganges) and nonaccumulator plants: lettuce, radish and alfalfa, Environ. Toxicol. 23 (2008) 607–616.
[42] M.L. López, J.R. Peralta-Videa, J.G. Parsons, T. Benítez, J.L. Gardea-Torresdey, Gibberellic acid, kinetin and the mixture indole-3-acetic acid-kinetin assisted with EDTA-induced lead hyperaccumulation in alfalfa plants, Environ. Technol. 41 (2007) 8165–8170.
[43] L.A. Del Río, L.M. Sandalio, J. Yanez, M. Gómez, Induction of a manganese-containing superoxide-dismutase in leaves of *Pisum Sativum* L. by high nutrient levels of zinc and manganese, J. Inorg. Biochem. 24 (1985) 25–34.
[44] T. Ducic, A. Polle, Transport and detoxification of manganese and copper in plants, Braz. J. Plant Physiol. 17 (2005) 103–112.
[45] P.R. Escuredo, F.R. Minchin, Y. Gogorcena, I. IturbeOrmaetxe, R.V. Klucas, M. Becana, Involvement of activated oxygen in nitrate-induced senescence of pea root modules, Plant Physiol. 110 (1996) 1187–1195.
[46] Y. Gogorcena, A.J. Gordon, P.R. Escuredo, F.R. Minchin, J.F. Witty, J.F. Moran, M. Becana, N-2 fixation, carbon metabolism, and oxidative damage in nodules of dark-stressed common bean plants, Plant Physiol. 113 (1997) 1193–1201.
[47] B.D. McKersie, Y. Chen, M. de Beus, S.R. Bowley, D. Bowler, D. Inzé, K. D'Hallium, J. Botterman, Superoxide dismutase enhances tolerance of freezing stress in transgenic alfalfa (*Medicago sativa* L.), Plant Physiol. 103 (1993) 1155–1163.
[48] A.P. Vitoria, P.J. Lea, R.A. Azevedo, Antioxidant enzymes responses to cadmium in radish tissues, Phytochemistry 57 (2001) 701–710.
[49] J.L. Schnoor, L.A. Litch, S.C. Mc Cutcheon, N.L. Wolfe, L.H. Carreira, Phytoremediation of nutrient and organic contaminants, Environ. Sci. Technol. 29 (1995) 318–323.
[50] K.V.S.K. Prasad, P.P. Saradhi, P. Sharmila, Concerted action of antioxidant enzymes and curtailed growth under zinc toxicity in *Brassica juncea*, Environ. Exp. Bot. 42 (1999) 1–10.
[51] S. Davletova, L. Rizhsky, H.J. Liang, S.Q. Zhong, D.J. Oliver, J. Coutu, V. Shulaev, K. Schlauch, R. Mittler, Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of Arabidopsis, Plant Cell 17 (2005) 268–281.
[52] C.H. Foyer, G. Noctor, Oxygen processing in photosynthesis: regulation and signalling, New Phytol. 146 (2000) 359–388.
[53] A. Puppo, K. Groten, F. Bastian, R. Carzaniga, M. Soussi, M.M. Lucas, M.R. De Felipe, J. Harrison, H. Vanacker, C.H. Foyer, Legume nodule senescence: roles for redox and hormone signalling in the orchestration of the natural aging process, New Phytol. 165 (2005) 683–701.
[54] G. Innocenti, C. Pucciariello, M. Le Gleuher, J. Hopkins, M. De Stefano, M. Delledonne, A. Puppo, E. Baudouin, P. Frendo, Glutathione synthesis is regulated by nitric oxide in *Medicago truncatula* roots, Planta 225 (2007) 1597–1602.
[55] C. Frova, The plant glutathione transferase gene family: genomic structure, functions, expression and evolution, Physiol. Plant 119 (2003) 469–479.
[56] M. Becher, I.N. Talke, L. Krall, U. Kramer, Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*, Plant J. 37 (2004) 251–268.
[57] M. Inouhe, Phytochelatins, Braz. J. Plant Physiol. 17 (2005) 65–78.
[58] E. Grill, E.L. Winnacker, M.H. Zenk, Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins, Proc. Natl. Acad. Sci. U.S.A. 84 (1987) 439–443.